REMARKS

Claims 1-17 are pending.

The Examiner has maintained the rejection of claims 1-6 and 14-17 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* et al. (U.S. Patent No. 6,207,387) in view of either Ehrlich (Bicheimica et Biophysica Acta 395:109-119, 1975) or Hua (Gov. Rep. Announce. Index US, 88, No.18, Abstract No. 847,050, 1988), and in further view of either Tyagi et al (U.S. Patent No. 6,150,097, Nov. 2000) or Coull (U.S. Patent No. 6,355,421, March 2002). Applicant respectfully again traverses this rejection and has provided further argument.

The Examiner has maintained the rejection of claims 7, 10, 12 and 13 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* in view of either Ehrlich or Hua, and in further view of either Tyagi et al or Coull, and further in view of Herman et al. (U.S. Patent 6,265,171, July 2001). Applicant respectfully again traverses this rejection as described herein.

The Examiner has maintained the rejection of claims 7 and 8 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* in view of either Ehrlich or Hua, and in further view of either Tyagi et al or Coull, and further in view of Kay et al. (Leukemia and Lymphoma, 24:211-220, 1997). Applicant again respectfully traverses this rejection as described herein.

The Examiner has rejected claims 1-17 on new grounds, under 35 U.S.C. § 112, first paragraph as allegedly containing new matter in view of recitation of "sufficient spatial proximity" and "methylated CpG-containing variants." Applicant has amended the claims and provided aguments to obviate this rejection.

No new matter has been added.

Rejections under 35 U.S.C. § 103(a)

Elsas et al, in view of Ehrlich or Hua, and in further view of Tyagi or Coull

The Examiner has maintained the rejection of claims 1-6 and 14-17 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* et al. (U.S. Patent No. 6,207,387) in view of either Ehrlich (Bicheimica et Biophysica Acta 395:109-119, 1975) or Hua (Gov. Rep. Announce.

Index US, 88, No.18, Abstract No. 847,050, 1988), and in further view of either Tyagi et al (U.S. Patent No. 6,150,097, Nov. 2000) or Coull (U.S. Patent No. 6,355,421, March 2002).

Applicant again respectfully traverses this rejection as described herein, based on the fact that no *prima facie* case of obviousness is supportable with these asserted references, alone or in combination. Applicant has further clarified the arguments of records and has provided additional supportive arguments. Again, the asserted references actually *teach away* from the presently claimed aspects of the invention.

The present claims:

The presently claimed aspects of the invention are drawn to methods for detecting methylated nucleic acids comprising: contacting (hybridizing) a target nucleic acid sample suspected of containing methylated nucleotides with at least one oligonucleotide probe (molecular beacon-type) comprising first and second stems having fluorophore and a quencher moieties, respectively, and loop sequence having a region of nucleotides complementary to at least a region of the nucleic acid that is susceptible to methylation, wherein-the nucleotides forming the first stem are capable of moving into spatial proximity with the nucleotides forming the second stem when the probe is dissociated from the nucleic acid sample; altering the hybridization conditions such that the oligonucleotide probe dissociates from unmethylated nucleic acids but remains hybridized to methylated nucleic acids; and measuring the change in fluorescence, wherein an increase in fluorescence indicates methylated nucleotides in said nucleic acid sample.

Significantly, the claimed aspects encompass the novel and non-obvious conception and appreciation that the oligonucleotide probe (non-methylated oligo) melting temperature *differential* between methylated and unmethylated target sequences, is capable, adequate or sufficient for effective CpG dinucleotide methylation detection using a non-methylated target specific 'loop' sequence in the context of a molecular beacon type probe. That the melting temperature differential would reasonably have such a utility and/or sufficiency is not suggested by the asserted art and teachings, alone or in combination because the asserted prior art structures (relatively long, double stranded, fully methylated sequences) are fundamentally different than the

present stuctures (non-methylated oligomer hybridizing over a relatively limited region to a methylated or unmethylated target sequence), and even if there were a suggestion to combine the references and teachings as urged by the Examiner, there could have been no reasonable expectation of success because of these fundamental structural differences.

The asserted references:

Elsas

As construed by the Examiner, Elsas teaches detection of mutations by determining the melting temperature between oligonucleotide probes and amplified genomic DNA sequences. Significantly, however, Elsas do not teach molecular beacon type probes, and do not teach application to methylated DNA nor thermodynamic characteristics of methylated DNA. Moreover, the melting temperature teachings are limited to detecting sequences differing by at least one nucleotide (*i.e.*, a **mismatch**). Therefore, differential melting in Elas is not based on a simple base modification as in the present case, but is rather based on the presence of at least one base mismatch between the probe and target in the detected target sequence. Such Tm differences, as recognized in the art, are rather substantial. For example, in Elsas' Example 3, the Tm differential between the wild-type and the galactosemia-positive mutant (Q 188R) allele is 9°C (*i.e.*, the Tm for the hybrid of amplified mutant DNA with the wild-type probe is 56°C, whereas the Tm for the hybrid of amplified mutant DNA hybrid with the mutant allele is 65°C (Elsas at column 11, lines 5-11).

Erlich

As construed by the Examiner, Erlich teaches that the melting temperature differential between Xanthomonas phage XP-12 DNA containing 5-methylcytosine replacing cytosine in ALL cytosine positions (*i.e.*, all positions on both strands over the full strand length; in fact, it is the only DNA known to have all cytosines methylated) and normal DNA with the same adenine plus thymine percentage is 6.1°C.

Applicant points out, however, that Ehrlich does not: (i) compare XP-12 DNA with unmethylated XP-12 DNA; (ii) compare melting of XP-12 methylated on only one strand with

unmethylated XP-12 DNA; and (iii) does not, and could not possibly teach the degree/amount/extent that one, or a limited number of methylated cytosines would contribute, if at all, to such a differential Tm, because, as will be argued in more detail below, the Tm difference measured by Erlich reflects an ***averaged*** Tm difference over the entire length of the XP-12 DNA; that is, what is being measured is not melting of a particular limited region of one strand of the XP-12 DNA hybridized to a non-methylated oligo, but rather the *averaged* melting across the entire length of the double-stranded XP-12 DNA. As will be discussed in more detail below, identification in the art of such an averaged differential Tm between fully methylated double-stranded DNA over the entire length does not allow prediction with any reasonable expectation of success that that there will be a differential Tm between an unmethylated oligomer hybridizing to a limited target region of single strand of a DNA molecule that is methylated at its CpG positions (partially or fully). This is because of the unpredictability of 'nearest neighbor effects' that are well known in the art.

Erlich, therefore, teaches nothing about efficient methods for the detection of methylated genomic sequences, and further teaches nothing about molecular beacon-type probes or the effective use thereof in the presently claimed context.

Hua

Similarly, Hua teaches that the melting temperature of completely methylated **Z**-DNA is 7K higher than unmethylated **B**-form DNA. Significantly, however, Hua is not just comparing the effects of methylation versus non-methylation, but rather is comparing the methylated form of one unique DNA form/structure (Z-DNA) with the unmethylated form of another DNA (B-form) structure under very specific salt conditions. Therefore, it is impossible to deduce/distinguish methylation effects on Tm from DNA structure effects on Tm. Additionally, Hua like Ehrlich, teaches differential Tm in the context of melting of a DNA duplex completely methylated on both strands and is impossible from the data of Hua to assess the melting of a duplex methylated on only one strand; that is the Tm difference measured by Erlich reflects an ***averaged*** Tm difference over the entire length of the DNA, and what is being measured is not melting of a particular

limited region of one strand of the methylated DNA hybridized to non-methylated oligo, but rather the *averaged* melting across the entire length of the double-stranded DNA. Therefore, aside from the structural differences in the compared DNAs, Hua teaches nothing about the degree/amount/extent that one, or a limited number of methylated positions would contribute to such a differential Tm, or about efficient methods for the detection of methylated genomic sequences, and further teaches nothing about molecular beacon-type probes or the effective use thereof in the presently claimed context. As will be discussed in more detail below, identification in the art of such an averaged differential Tm between fully methylated double-stranded DNA over the entire length does not allow prediction with any reasonable expectation of success that that there will be a differential Tm between an unmethylated oligomer hybridizing to a limited target region of single strand of a DNA molecule that is methylated at its CpG positions (partially or fully). This is because of the unpredictability of 'nearest neighbor effects' that are well known in the art.

Tyagi

Tyagi et al., consistent with the Examiner's interpretation, "discovered that in certain Molecular Beacon probes, a pair of labels "touches" when the probe is not hybridized to a target," so "that the absorption spectrum is significantly altered." (Tygai at column 5 lines 31-34). Therefore, Tyagi teaches quenching in the context of FRET pairs. Tyagi, however, teaches nothing about efficient methods for the detection of methylated genomic sequences, and further teaches nothing about the effective use of molecular beacon-type probes in the presently claimed context of detection of methylated nucleic acid sequences.

Coull

Finally, Coull et al., teach peptide nucleic acid (non-naturally occurring polyamide) molecular beacons that have advantageous properties (less susceptible to degradation, etc.). Coull, however, teaches nothing about efficient methods for the detection of methylated genomic sequences, and further teaches nothing about the effective use of molecular beacon-type probes in the presently claimed context of detection of methylated nucleic acid sequences.

Applicable law:

To establish a *prima facie* case of obviousness there must be: (i) a suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (ii) a reasonable expectation of success; and (iii) the prior art reference(s) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure (*In re Vaeck,* 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991); and see MPEP §§ 2143-2143.03). Therefore, to support a conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references. Moreover, there can be no reasonable expectation of success where the art, alone or in combination, *teaches away* from the invention.

As discussed herein, applicant respectfully contends that no *prima facie* case of obviousness can be made in the present case, because there is no suggestion in Elsas, alone or in combination with any of the other cited references, to provide for the instant novel methods, which provide for effective detection of methylated nucleic acid sequences based on the use of molecular beacon-type probes in the context of Tm measurements. Identification in the art of an ***averaged*** differential Tm between fully methylated double-stranded DNA over the entire length does not allow prediction with any reasonable expectation of success that that there will be a differential Tm between an unmethylated oligomer hybridizing to a limited target region of single strand of a DNA molecule that is methylated at its CpG positions (partially or fully) to provide for an effective methylation assay. This is because of the unpredictability of 'nearest neighbor effects' that are well known in the art.

Analysis:

Applicant's arguments already of record are reaffirmed and reasserted herein. The asserted

art alone or in combination simply does not teach or suggest the claimed combination, and no such teaching or suggestion in these references has in fact been cited by the Examiner. Moreover, *arguendo*, even if there were such suggestions to combine, there would have been no reasonable expectation of success in applying the teachings Ehrlich and Hua (with respect to the differences in Tm between methylated and unmethylated DNA) to the teachings about allele discrimination using molecular beacon-type probes to arrive at the presently claimed invention.

No teaching or suggestion. As discussed above in detail with respect to the asserted references, alone or in combination, there is no teaching or embodied expectation as to the degree/extent/amount that one or a limited number of cytosine methylations would make to a differential Tm in the presently claimed context. The cited references teach ***averaged*** Tm measurements across the full-length of completely methylated (on both strands) duplex DNA sequences, and also teaches ***averaged*** Tm measurements across the full-length of DNAs having different structures (Hua) in addition to being methylated or not. Moreover, as discussed above, Elsas teaches at least one base *mismatch,* associated with a significant (9°C) effect on Tm. Based on the cited references, it would certainly not have been obvious that the Tm differentials associated with cytosine methyation would have been sufficient to enable effective detection of methylated nucleic acids in the context of Tm measurement using molecular beacon-type probes. As discussed below in more detail, identification in the art of an ***averaged*** differential Tm between fully methylated double-stranded DNA over the entire length does not allow prediction with any reasonable expectation of success that that there will be a differential Tm between an unmethylated oligomer hybridizing to a limited target region of single strand of a DNA molecule that is methylated at its CpG positions (partially or fully) to provide for an effective methylation assay. The presently claimed molecular beacon 'loop' probe sequences are not themselves methylated, so that only the target strand of the DNA is methylaed or not (*i.e.*, the complementary target region of the detected genomic strand is methylated or not).

No reasonable expectation of success. If anything, Elsas, Ehrlich and Hua collectively *teach away* from the present invention by teaching that the differential effects on Tm of

methylation are measureable only in an ***averaged*** fashion across the full length of a fully methylated DNA duplex structure. There is simply no basis given the teachings of the asserted art to predict with a reasonable expectation of success, that any potential Tm effects would allow for the presently claimed effective detection assays that are based on a differential Tm between an unmethylated oligomer loop sequence hybridizing to a limited target region of single strand of a DNA molecule that is methylated or not at its CpG positions. In such situations, potential nearest neighbor effects would have made it impossible to predict with reasonable expectation that the presently claimed assays would work.

'Nearest neighbor effects'. As appreciated in the art, "melting temperature" or "Tm" refers the temperature at which a complementary complex of nucleic acids, usually double-stranded, becomes half dissociated into single strands. As appreciated in the art, a simple estimate of the Tm value may be calculated using the equation Tm = 81.5 + 0.41(%G + C), when a nucleic acid is in aqueous solution at 1 M NaCl. More accurate Tm calculations can be made using the base pair thermodynamics of a "nearest-neighbors" approach (Breslauer K.J. et al (1986) *Proc. Natl. Acad. Sci. USA*, **83**: 3746-3750; SantaLucia J. Jr. (1998) *Proc. Natl. Acad. Sci. USA*, **95**: 1460-1465; both attached hereto with supplementary IDS) The energetic effect of the bases on DNA duplex stability depends on the nearest neighbor bases as has been described in Breslauer. Energy contribution of every natural base in a sequence varies considerably depending on the nearest neighbors (*Id*). Significantly, however, nearest-neighbor effects have only been determined for natural bases. 5-methyl-C is not considered to be a natural base and there are <u>no</u> reports on measuring nearest neighbor contributions to 5-MeC.

The situation is further complicated by the fact that certain nucleotides were already known to be stabilizing or destabilizing depending on the neighboring sequence context. For example, as appreciated in the art, 2,6-Diaminopurine is a structural analog of adenosine, and introduction of an additional amino group at the C2 heterocyclic atom leads to formation of three hydrogen bonds with thymidine and this strongly stabilizes the duplex. However, <u>2,6-diaminopurine is not always a stabilizing agent</u> and its thermodynamic effect on duplex DNA

depends on the nearest neighbors; that is, in certain neighborhoods 2,6-diaminopurine is destabilizing modification (Cheong, C., Tinoco, I. and Chollet, A. (1988) Thermodynamic studies of base pairing involving 2,6-diaminopurine. *Nucl. Acids Res.*, **16**: 5115-5122; attached hereto with supplementary IDS). The point is that nearest neighbor effects were not characterized for 5-MeC in the art, and no one could have predicted what such effects would be in the context of the presently claimed assay methods; namely, a differential Tm between an unmethylated oligomer loop sequence hybridizing to a limited target region of single strand of a DNA molecule that is methylated or not at its CpG positions. In such situations, potential nearest neighbor effects would have made it impossible to predict success with reasonable expectation.

In similar fashion, recognition in the art (as in the presently asserted references) that one can determine a differential Tm based on measurements averaged over the entire length of a relatively long double-stranded, fully methylated (both strands) DNA molecule could not have precluded the possibility that 5-MeC would have a negative effect in the context of certain neighboring bases, and such effects would not be average out over the relatively short distances of the molecular beacon probes. While the 5'-CpG-3' sequence is fixed with a neighboring G on the 3' side, the 5' base is unknown and the 5'-neighbor is the most important in predicting the nearest neighbor effects (Breslauer K.J. supra).

As discussed above, the asserted art <u>only</u> teaches that methylation effects one Tm are measureable in an ***averaged*** fashion across the full length of a fully methylated DNA duplex structure. The prior art does not even teach that such differential effects could be measured in the context of a full-length duplex sequence wherein only one of the strands was methyltion, much less teach that such differential effects would be reasonably expected in the presently claimed context. Significantly, there is no teaching or suggestion that there would be a differential Tm between an unmethylated oligomer loop sequence hybriding to a limited target region of one strand of a DNA molecule that is methylated or not on that strand at its CpG positions.

Applicant, therefore, respectfully requests withdrawal of the Examiner's 35 U.S.C. § 103(a) rejections of claims 1-6 and 14-17.

Elsas et al, in view of Ehrlich or Hua, in further view of Tyagi or Coull, and in further view of Herman et al.

The Examiner has maintained the rejectioin of claims 7, 10, 12 and 13 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* in view of either Ehrlich or Hua, and in further view of either Tyagi et al or Coull, and further in view of Herman et al. (U.S. Patent 6,265,171, July 2001).

The Examiner states that while "Elsas, Ehrlich, Hua, Tyagi or Coull do not teach detecting methylation in GST pi or calcitonin which is differentially methylated in cancer versus normal state," Herman nonetheless "teaches numerous genes which are differentaiilly methylated at CpG islands in neoplastic versus normal tissue," and that "CpG island differential methylation can be detected in prostate cancer."

Applicant respectfully traverses this rejection based on the arguments discussed in detail above with respect to the Examiner's rejection of claims 1-6 and 14-17. The combination of cited references does not anticipate independent claim 1 or render it obvious.

Applicant, therefore, respectfully requests withdrawal of the Examiner's 35 U.S.C. § 103(a) rejections of claims 7, 10, 12 and 13.

Applicant notes that the Examiner, despite the reference to differentially methylated CpG island sequences and prostate cancer, has made no specific allegation with respect to the teachings, if any, of Herman with respect to differential GSTpi and calcitonin methylation and prostate cancer.

Elsas et al, in view of Ehrlich or Hua, in further view of Tyagi or Coull, and in further view of Kay et al.

The Examiner has maintained the rejection of claims 7 and 8 under 35 U.S.C. § 103(a), as being allegedly unpatentable over *Elsas* in view of either Ehrlich or Hua, and in further view of

either Tyagi et al or Coull, and further in view of Kay et al. (Leukemia and Lymphoma, 24:211-220, 1997). Applicant respectfully traverses this rejection as described herein.

The Examiner states that while "Elsas, Ehrlich, Hua, Tyagi or Coull do not teach differential methylation in Myf-3 which is differentially expressed in cancer versus normal state," Kay nonetheless teaches "detecting methylation in Myf-3, which is differentially expressed in cancer versus a normal state" ("hypermethylated in non-Hodgkins lymphoma").

Applicant respectfully traverses this rejection based on the arguments discussed in detail above with respect to the Examiner's rejection of claims 1-6 and 14-17. The combination of cited references does not anticipate independent claim 1 or render it obvious.

Applicant, therefore, respectfully requests withdrawal of the Examiner's 35 U.S.C. § 103(a) rejections of claims 7 and 8.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 1-17 on new grounds, under 35 U.S.C. § 112, first paragraph as allegedly containing new matter in view of recitation of "sufficient spatial proximity" and "methylated CpG-containing variants."

Specifically, the Examiner states, with respect to claim 1, that recitation of "sufficient spatial proximity" is new matter. Applicants disagree, but have nonetheless deleted the word "sufficient."

Additionally, the Examiner states, with respect to claims 9 and 11, that recitation of "methylated CpG-containing variants" is new matter. Applicant respectfully traverses this rejection, but has nonetheless amended the claims.

Original claim 1 and the originally filed specification (e.g., at page 4, ll. 10-11) recite "a loop sequence having a region of nucleotides complementary to at least a region of the nucleic acid sample, which region is susceptible to methylation...." Therefore, the specification teaches explicitly that the loop sequences are designed to be complementary to (hybridize to) sequences that are susceptible to methylation; that is sequences that can be methylated or not. The

specification also teaches that SEQ ID NOS:1-5 are such complementary sequences that are susceptible to methylation. Therefore, the specification provides adequate support for recitation of "methylated CpG-containing variants."

Applicant has nonetheless amended claim 9 to recite " wherein the nucleic acid sequence that undergoes methylation is ~~the loop sequence is complementary to at least one of the sequences~~ selected form the group consisting of SEQ ID NOS:1-3 ~~and methylated CpG-containing variants thereof~~. Conforming amendments have been made to claim 11. Support and antecedent basis for the amendments is found in the recitations of claims 8 and 11, respectively.

No new matter has been added.

CONCLUSION

Applicant respectfully requests entry of the present Response and Amendment, and allowance of all claims as presented and amended herein.

The Examiner is encouraged to phone applicant's attorney, Barry L. Davison, to resolve any outstanding issues and expedite allowance of this application.

Respectfully submitted,

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